BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 20(1), 57-61 (2012)

Baicalein Attenuates Oxidative Stress-Induced Expression of Matrix Metalloproteinase-1 by Regulating the ERK/JNK/AP-1 Pathway in Human Keratinocytes

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Abstract

The matrix metalloproteinase (MMP) family is involved in the breakdown of the extracellular matrix during normal physiological processes such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes such as pathological aging, arthritis, and metastasis. Oxidative conditions generate reactive oxygen species (ROS) (e.g., hydrogen peroxide [H_2O_2]) in cells, which subsequently induce the synthesis of matrix metalloproteinase-1 (MMP-1). MMP-1, an interstitial collagenase, in turn stimulates an aging phenomenon. In this study, baicalein (5,6,7-trihydroxyflavone) was investigated for its *in vitro* activity against H_2O_2-induced damage using a human skin keratinocyte model. Baicalein pretreatment significantly inhibited H_2O_2-induced up-regulation of MMP-1 mRNA, MMP-1 protein expression and MMP-1 activity in cultured HaCaT keratinocytes. In addition, baicalein decreased the transcriptional activity of activator protein-1 (AP-1) and the expression of c-Fos and c-Jun, both components of the heterodimeric AP-1 transcription factor. Furthermore, baicalein reduced phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK), which are upstream of the AP-1 transcription factor. The results of this study suggest that baicalein is involved in the inhibition of oxidative stress-induced expression of MMP-1 via inactivation of the ERK/JNK/AP-1 signaling pathway.

Key Words: Baicalein, Matrix metalloproteinase, Oxidative stress, Reactive oxygen species, Hydrogen peroxide, Signal transduction

INTRODUCTION

The degradation of the extracellular matrix (ECM) is essential for embryonic development, morphogenesis, reproduction, and tissue remodeling. The family of matrix metalloproteinases (MMPs) in general, and matrix metalloproteinase-1 (MMP-1) in particular, play a central role in these processes. MMP-1, or interstitial collagenase, is a secreted protein that contributes to the etiology of many age-related degenerative diseases (Jacob, 2003; Kähäri and Saarialho-Kere, 1997). MMP-1 is a prominently involved in the proteolytic release and activation of growth factors, cytokines, and signaling peptides, which also have the potential to modulate the senescent microenvironment (Dasgupta *et al.*, 2010).

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) readily undergo reactions with thiol groups and may, thus, participate in a common mechanisms underlying the activation of several different MMPs, including MMP-1 (Rajagopalan *et al.*, 2003). H_2O_2 regulates the activity of critical signaling molecules, leading to augmented MMP-1 expression in human skin cells (Brenneisen *et al.*, 1997). Furthermore, the redox activation of c-Jun-N-terminal kinase (JNK) controls the activity of the activator protein-1 (AP-1) transcription factor, resulting in an age-dependent increase in MMP-1 expression (Dasgupta *et al.*, 2010). Moreover, oxidative stress stimulates the activity of extracellular signal-regulated kinase (ERK), which are also important for the regulation of MMP-1 expression. Blockade of the ERK pathway was found to abrogate the Ras- and serum-induced stimulation of the MMP-1 promoter, indicating a role for ERK in the transcriptional regulation of MMP-1 (Frost *et al.*, 1994). These studies suggest that the ERK/JNK/AP-1 pathway may be the major activator of MMP-1 gene and protein expression.

A number of studies demonstrate inhibition of MMP-1 up-

www.biomolther.org

Open Access http://dx.doi.org/10.4062/biomolther.2012.20.1.057

pISSN: 1976-9148 eISSN: 2005-4483
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Received Dec 1, 2011 **Revised** Dec 24, 2011 **Accepted** Dec 27, 2011

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regulation by antioxidants (Brenneisen *et al.*, 2002; Nelson and Melendez, 2004), including N-acetylcysteine (NAC), a precursor of glutathione (Kheradmand *et al.*, 1998; Cho *et al.*, 2006; Zaw *et al.*, 2006). Previous work from our group demonstrated that triphlorethol-A, an antioxidant, participates in the modulation of MMP-1 level in cultured cells (Kang *et al.*, 2008). These data provide further support for the ability of ROS to initiate signaling pathways that lead to MMP-1 induction.

Baicalein (5,6,7-trihydroxyflavone) is a flavonoid derived from the roots of *Scutellaria baicalensis*. Baicalein attenuates oxidative stress and protects cardiomyocytes from lethal oxidant damage in an ischemia-reperfusion model (Shao *et al.*, 1999; Shao *et al.*, 2002). In addition, our recent work showed that baicalein ameliorated mitochondrial oxidative stress by activating nuclear factor (erythroid-derived 2)-like 2-mediated induction of manganese superoxide dismutase (Lee *et al.*, 2011) and protected cellular components against oxidative damage by scavenging ROS and inhibiting apoptosis (Kang *et al.*, 2012). On the other hand, the protective effect of baicalein against ROS-associated stimulation of MMP-1 expression has not been investigated. Therefore, the current study focused on the ability of baicalein to safeguard cultured human keratinocytes against H_2O_2-mediated MMP-1 induction and investigated the possible underlying molecular mechanisms.

MATERIALS AND METHODS

Cell culture

Human keratinocytes (HaCaT cells) were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 U/ml). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Reagents

Baicalein (Fig. 1) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The primary MMP-1 antibody was purchased from Epitomics, Inc. (Burlingame, CA, USA). Primary antibodies against phospho MEK1 (mitogen-activated protein (MAP) kinase kinase1), MEK1, phospho ERK1/2, ERK2, phospho SEK1 (stress-activated protein kinase (SAPK)/ERK kinase1), SEK1, phospho JNK1/2, JNK1/2, c-Fos, and phospho c-Jun were purchased from Cell Signaling Technology (Beverly, MA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were seeded in a 96-well plate at a density of 1.5×10^5 cells/well. Sixteen hours after plating, the cells were treated with baicalein at a concentration of 5 μg/ml. After 30 min, H_2O_2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. Total RNA was isolated from the cells using an easy-BLUE RNA extraction kit (Intron, Daegeon, Korea). The PCR conditions for MMP-1 and the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were as follows: 35 cycles of 94°C for 45 sec; 52°C for 1 min; and 72°C for 1 min. The forward and reverse primer pairs for MMP-1 and GADPH (Bionics, Seoul, Korea) were as follows: human MMP-1, 5′-GAGGAAATCTTGCTCAT-3′ and 5′-CTCAGAAAGAGCAGCATC-3′; and human GAPDH, 5′-AAGGTCGGAGTCAACGGATTT-3′ and 5′-GCAGTGAGGGTCTCTCTCCT-3′. The amplified products were resolved by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

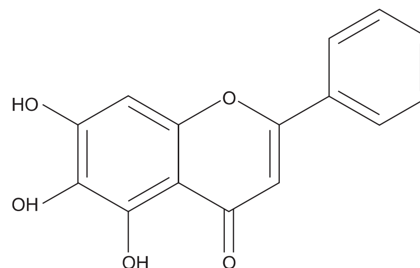

Fig. 1. Chemical structure of baicalein (5,6,7-trihydroxyflavone).

Western blot analysis

Cells were seeded in a 96-well plate at a density of 1.5×10^5 cells/well. Sixteen hours after plating, the cells were treated with baicalein at a concentration of 5 μg/ml. After 30 min, H_2O_2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. Cells were then lysed in lysis buffer (100 μl; 120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP-40). Aliquots of the lysates (40 μg protein) were boiled for 5 min and electrophoresed in a 10% SDS-polyacrylamide gel. The proteins in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), and the membranes were subsequently incubated with the primary antibodies. The membranes were further incubated with secondary anti-IgG-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA) followed by exposure to X-ray film. The protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, UK).

Determination of MMP-1 activity

Cells were seeded in a 96-well plate at a density of 1.5×10^5 cells/well. Sixteen hours after plating, the cells were treated with baicalein at indicated concentrations or pretreated with 1 mM of N-acetyl cysteine (NAC) for 1 h. After 30 min, H_2O_2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. MMP-1 activity was determined using a Fluorokine® E human active MMP-1 fluorescent assay (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions, which uses a quenched fluorogenic substrate. Production of the fluorescent cleavage product was determined using a fluorescence plate reader set (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 320 nm and emission wavelength of 405 nm.

Transient transfection and AP-1 luciferase assay

Cells were transiently transfected with a plasmid harboring the AP-1 promoter using Lipofectamine™ 2000, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Following an overnight transfection, cells were treated with baicalein (5 μg/ml). After an additional incubation for 1 h, cells were treated with H_2O_2 (1 mM). After 6 h, the cells were washed twice with PBS and then lysed with a passive lysis buffer (Promega, Madison, WI, USA). Following vortex-mixing and centrifugation at 12,000×g for 30 sec at 4°C, the supernatant was stored −70°C until use in the luciferase assay. After mixing the cell extract (20 μl) with the luciferase assay substrate reagent (100 μl) at room temperature, the mixture was

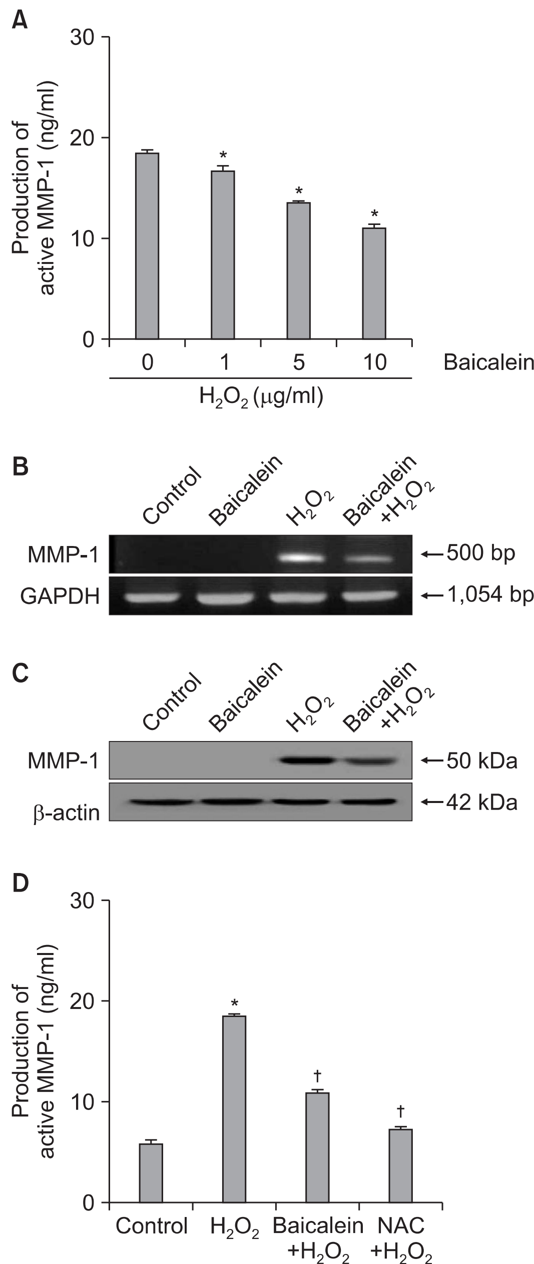

Fig. 2. Effects of baicalein on H_2O_2-induced MMP-1 mRNA expression, protein expression, and activity. (A) Cells were treated with baicalein at indicated concentrations. After 30 min, H_2O_2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. MMP-1 activity was measured using a fluorimetric ELISA assay, as described in Materials and Methods. Each bar represents the mean ± the standard error from triplicate experiments. *Significantly different from H_2O_2 treatment (p<0.05). (B) Total RNA was extracted, and MMP-1 mRNA expression was analyzed by RT-PCR. The GAPDH band is shown to confirm the integrity and equal loading of RNA. (C) Cell lysates were electrophoresed in SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The expression of the MMP-1 protein was detected with a primary antibody specific for MMP-1. The actin band is shown to confirm equal loading of protein. (D) Cells were treated with baicalein or NAC and after 30 min, H_2O_2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. MMP-1 activity was measured using a fluorimetric ELISA assay, as described in Materials and Methods. Each bar represents the mean ± the standard error from triplicate experiments. *Significantly different from untreated control (p<0.05). †Significantly different from H_2O_2 treatment (p<0.05).

placed in an illuminometer to measure the light produced. The amount of light produced provided a measure of AP-1 luciferase activity, and hence, AP-1 transcriptional activity.

Statistical analysis

All measurements were performed in triplicate, and all data represent the mean ± the standard error. The results were subjected to an analysis of variance (ANOVA) using Tukey's test to analyze differences between the means; p<0.05 was considered to be significant.

RESULTS

Reduction of H_2O_2-induced MMP-1 expression and activity by baicalein treatment

H_2O_2 and other ROS induce oxidative stress in many types of cells, including human keratinocytes (O'Toole *et al.*, 1996; Shvedova *et al.*, 2003). In particular, excessive amounts of ROS can stimulate mRNA and protein expression of MMP-1, a hallmark of oxidative stress (Brenneisen *et al.*, 1997). Baicalein treatment at 1, 5, and 10 μg/ml significantly prevented the production of active MMP-1 in a concentration-dependent manner (Fig. 2A), and 5 μg/ml of baicalein was determined as the optimal concentration for further study. In the current study, H_2O_2 treatment markedly increased the levels of MMP-1 mRNA and protein in cultured HaCaT cells compared with those in control cells (no treatment), as evidenced by RT-PCR (Fig. 2B) and Western blot (Fig. 2C) analyses. However, baicalein pretreatment inhibited the increased transcription of MMP-1 mRNA in H_2O_2-treated cells (Fig. 2B). Consistent with the RT-PCR results, baicalein also partially inhibited the H_2O_2-induced increase in MMP-1 protein expression (Fig. 2C). Moreover, H_2O_2 treatment increased the amount of active MMP-1 in HaCaT cells, whereas pretreatment with baicalein or an antioxidant NAC significantly prevented this increase (Fig. 2D). These results suggest that baicalein blocked H_2O_2-induced MMP-1 expression and activity.

Attenuation of H_2O_2-induced activation of AP-1 by baicalein

The AP-1 transcription factor exists as either a c-Jun/c-Jun homo-dimer or a c-Jun/c-Fos hetero-dimer and is involved in the activation of MMP genes (Glover and Harrison, 1995; Farrell *et al.*, 1989). H_2O_2 treatment stimulated the expression of both c-Fos and phospho c-Jun, while baicalein pretreatment decreased the extent of this stimulation (Fig. 3A). Baicalein pretreatment also significantly reduced H_2O_2-induced AP-1 transcriptional activity, as assessed by the AP-1 luciferase assay (Fig. 3B).

Reduction of H_2O_2-induced phosphorylation of MEK-ERK and SEK-JNK by baicalein

Members of the Fos and Jun families of transcription factors are regulated by members of the MAP kinase family, particularly ERK and JNK (Cano *et al.*, 1994; Murphy *et al.*, 2002; Bogoyevitch *et al.*, 2010). As shown Fig. 4A, phospho ERK1/2 and phospho MEK1, which is upstream of ERK1/2, were markedly increased by H_2O_2 treatment. Baicalein reduced the amount of phospho ERK1/2 and phospho MEK1 induced by H_2O_2 (Fig. 4A). Furthermore, baicalein pretreatment attenuated the H_2O_2-mediated up-regulation of phospho JNK1/2 and

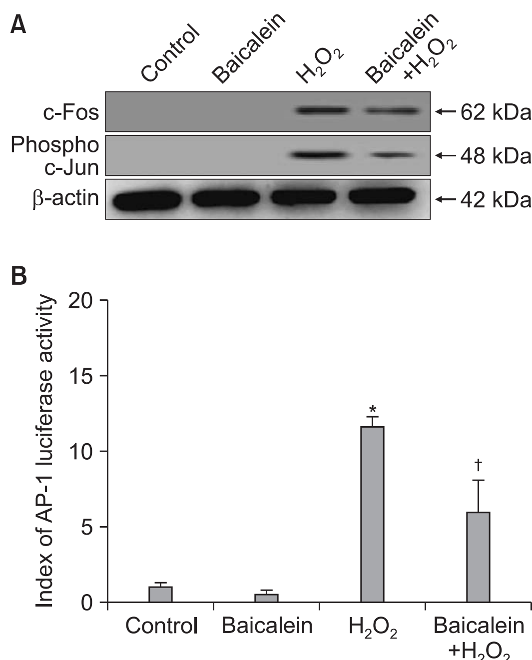

Fig. 3. Effects of baicalein on AP-1 expression and transcriptional activity. (A) Cell lysates were electrophoresed in SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and immunoblotted using antibodies specific for c-Fos and phospho c-Jun. The actin band is shown as a loading control. (B) The transcriptional activity of AP-1 was assessed using an AP-1 luciferase assay as described in Materials and Methods. Each bar represents the mean ± the standard error in triplicate experiments. *Significantly different from control (p<0.05). †Significantly different from H_2O_2 treatment (p<0.05).

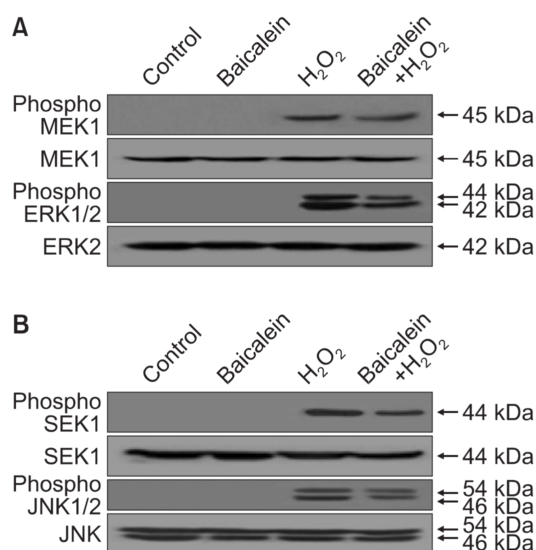

Fig. 4. Effects of baicalein on ERK and JNK signal transduction. (A) Cell lysates were electrophoresed in SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and immunoblotted using primary antibodies specific for (A) phospho MEK1, MEK1, phospho ERK1/2, and ERK2, and (B) phospho SEK1, SEK1, phospho JNK1/2, and JNK1/2.

phospho SEK1, which is upstream of JNK1/2 (Fig. 4B). Taken together, the results of this study suggest that baicalein inhibits oxidative stress-induced expression and activity of MMP-1 by inactivating AP-1 and its associated signaling kinases.

DISCUSSION

ROS, including H_2O_2, hasten aging of the skin and contribute to processes such as ultraviolet B-induced photoaging, loss of elasticity, and wrinkling. These processes result, in large part, from the deterioration of the connective tissue matrix of the skin and, in particular, its collagen component. ROS produced in skin cells may contribute to the biological changes that are observed in the aging organ by accelerating the MMP-related ECM degradation system (Kawaguchi *et al.*, 1996). Furthermore, ROS are important intermediates in downstream signaling pathways that culminate in the induction of increased steady state levels of MMP-1 (Brenneisen *et al.*, 1997), an interstitial collagen-degrading enzyme.

In this study, H_2O_2 caused a pronounced increase in MMP-1 mRNA and protein expression in cultured human HaCaT keratinocytes. Importantly, H_2O_2 also increased the amount of active MMP-1. Pretreatment with baicalein, a flavonoid component of *Scutellaria baicalensis*, partially suppressed the actions of H_2O_2. Furthermore, H_2O_2 augmented the activity of the AP-1 transcription factor, which is formed by a c-Fos/c-Jun heterodimer, as well as the expression of c-Fos and phospho c-Jun. AP-1 binds to the 5' residues of the MMP-1 promoter, stimulating the expression of the MMP-1 gene (Mackay *et al.*, 1992; Lin *et al.*, 1993). Notably, baicalein pretreatment also reduced H_2O_2-induced c-Fos/phospho c-Jun expression and AP-1 transcriptional activity.

c-Fos and c-Jun, the key components of AP-1, are primarily regulated at the transcriptional level by phosphorylated MEK/ERK and SEK/JNK, respectively (Kieser *et al.*, 1997; Eliopoulos and Young, 1998; Favata *et al.*, 1998; Wu *et al.*, 2002). H_2O_2 enhanced the phosphorylation of MEK1/ERK1/2 and SEK1/JNK1/2, in keeping with its activation of AP-1. However, baicalein impaired the H_2O_2-induced phosphorylation of MEK1/ERK1/2 and SEK1/JNK1/2.

In conclusion, the results of the present study suggest that baicalein can safeguard HaCaT cells against oxidative stress-induced senescence-associated MMP-1 expression and activation via inhibition of the ERK/JNK/AP-1 pathway. These results have important implications for therapies designed to protect against premature aging of the skin and age-related skin disorders.

ACKNOWLEDGMENTS

This research was supported by a grant from the Korean Ministry of Knowledge and Economy (R0000445).

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